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Effect of IP-10/CXCR3 signaling pathway on rats with diabetic retinopathy

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ABSTRACT

This study aimed to investigate the effect of the interferon-inducible protein-10 (IP-10)/C-X-C motif chemokine receptor 3 (CXCR3) signaling pathway on rats with diabetic retinopathy. A total of 21 Sprague-Dawley rats were selected as the objects and divided into control (n=7), model (n=7) and inhibitor (n=7) groups. The rats in control group did not receive any treatment. The diabetic retinopathy model was established using streptozotocin and vascular endothelial growth factor in model group, while the rats in inhibitor group were treated with AMG 487, an inhibitor of the IP-10/CXCR3 signaling pathway, based on the treatment in model group. The changes in gene expression patterns in rats with diabetic retinopathy were screened by sequencing. After the differential genes were determined, the pathways mainly related to the complication were obtained *via* enrichment analysis. The expression of the IP-10/CXCR3 signaling pathway, the apoptotic cells and the expression of inflammatory molecules (IL-6, IL-12, TNF-α and IL-1β) in each group of rats were detected. It was shown in volcano plot that there were some differentially expressed genes (fold change >1.2, P<0.01) in retinal tissues of rats in control and model groups. Meanwhile, the heatmap displayed that there were great differences in the gene expression patterns between control and model groups, and the gene expressions of IP-10 and CXCR3 in model group were higher than those in control group (P<0.05). The differential genes in control and model groupwere enriched in such processes as the cAMP metabolic regulatory pathway, chemotaxis of immunocytes, proliferation of endothelial cells, response of cytokine receptors, apoptosis, mTOR signaling pathway and TGF-β-related signaling pathway. The mRNA expressions of IP-10 and CXCR3 in model group were higher than those in control group (P<0.05), while they were notably lower in inhibitor group than those in model group (P<0.05). Besides, the protein levels of IP-10 and CXCR3 were identical to the mRNA levels. The apoptotic cells were increased markedly in model group compared with those in control group (P<0.05) and inhibitor group (P<0.05). Model group exhibited higher expression levels of IL-6, TNF-α and IL-1β in retinal tissues than control group (P<0.05), while inhibitor group had distinctly lower expression levels of IL-6, TNF-α and IL-1β in retinal tissues than model group (P<0.05). The IP-10/CXCR3 signaling pathway can affect rats with diabetic retinopathy.

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Introduction

Diabetes mellitus is a kind of systemic metabolic disease characterized by long-term and chronic rise in blood glucose caused by various reasons (1). With the development of the social economy and the improvement of people's living standards, the morbidity rate of diabetes mellitus is increasing prominently (2). As an incurable disease, diabetes mellitus usually troubles patients all their life. It generally induces chronic and severe complications including diabetic nephropathy, microangiopathy and retinopathy, threatening the patient's life (3). Among them, diabetic retinopathy is an ocular complication of diabetes mellitus, manifested as hypopsia and even blindness induced by ocular microvascular lesions (4,5). The pathogenesis of diabetic retinopathy may be that high blood glucose and lipid levels in patients lead to endothelial cell injury, local hypoxia and occlusion in ocular blood vessels, resulting in vascular damage and disturbance of blood supply of the eyes (6,7). Studies have demonstrated that the incidence of diabetic retinopathy is related to dysregulated expression of multiple intra-cellular pathways or molecules in organisms, including the NADPH oxidase-dependent pathway (8,9) and the SDF-1/C-X-C motif chemokine receptor 4 (CXCR4) pathway (10). However, the impact of the interferon-inducible protein-10 (IP-10)/CXCR3 signaling pathway on diabetic retinopathy remains unknown, and the specific pathogenesis of diabetic retinopathy has not been clarified yet. Hence, in this research, the rat model of diabetic retinopathy was established, and the changes in gene expression patterns in rats with diabetic retinopathy were detected by sequencing and other methods. After the differential genes were obtained, the pathways mainly associated with the complication were acquired *via* enrichment analysis and verified by experiments, so as to find and elaborate the influence of the IP-10/CXCR3 signaling pathway on diabetic retinopathy rats.

Materials and Methods

Selection and grouping of laboratory animals

A total of 21 adult male Sprague-Dawley rats (Specific Pathogen Free (SPF)-grade, 280±10 g) were selected as the objects and randomly assigned into control group

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(n=7), model group (n=7) and inhibitor group (n=7). This study was approved by the Animal Ethics Committee of Yancheng Third People's Hospital Animal Center.

Establishment of the rat model of diabetic retinopathy

All the rats were kept at a proper temperature and humidity, with clean paddings and adequate water sources. The rats in control group were raised normally, without any treatment. In model group, the model of diabetic mellitus was established by a single intraperitoneal injection of 60 mg/kg streptozocin (Sigma, St. Louis, MO, USA), and vascular endothelial growth factor was injected intravitreally to prepare the model of diabetic retinopathy 1 month later. Based on the treatment in model group, the rats in inhibitor group were subcutaneously injected with the IP-10/CXCR3 signaling pathway inhibitor AMG 487 (5 mg/kg) twice a day.

Transcriptome sequencing of retinal tissues

Transcriptome sequencing was adopted for the retinal tissues of rats in control group and model group, so as to find the changes in the expressions of genes and pathways related to diabetic retinopathy. After sacrificing the rats, the eyeballs were taken out to obtain the intact retinal tissues, from which total ribonucleic acids (RNAs) were extracted by the TRIzol method (Invitrogen, Carlsbad, CA, USA). Following concentration measurement, the samples were sent to Tianjin Biotechnology Co., Ltd. (Tianjin, China) for transcriptome sequencing as well as Genome Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses.

Real-time quantitative polymerase chain reaction (RT-qPCR)

The levels of IP-10, CXCR3, inflammatory molecules [interleukin-6 (IL-6), IL-12, tumor necrosis factor-alpha (TNF-α) and IL-1β] and internal reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the retinal tissues in control group, model group and inhibitor group were determined *via* RT-qPCR. The total RNAs in each group of retinal tissues were extracted using TRIzol method and then underwent PCR under the following conditions: 95°C for 5 min, (95°C for 35 s, 56°C for 45 s and 72°C for 30 s) ×40 cycles and 72°C for 5 min. The primers were synthesized and verified by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) (Table 1).

Western blotting (WB)

The protein expressions of IP-10, CXCR3 and internal reference GAPDH in the retinal tissues in control group, model group and inhibitor group were determined *via* WB assay. The retinal tissues in the three groups were lysed by protein lysis buffer containing phenylmethylsulfonyl fluoride (PMSF) (Beyotime, Shanghai, China) for 40 min and then centrifuged at 14,000 rpm for 20 min. Subsequently, the protein was subjected to electrophoresis, transferred onto a membrane, incubated with primary antibodies and secondary antibodies and exposed. After that, the expression levels of IP-10 and CXCR3 were detected by comparing them with that of internal reference GAPDH.

Detection of cell apoptosis

A TUNEL kit (Sigma-Aldrich, St. Louis, MO, USA) was utilized to detect the cell apoptosis according to the instructions. In short, the paraffin-embedded sections of retinal tissues in control group, model group and inhibitor group were placed in a wet box, incubated with terminal deoxynucleotidyl transferase in drops for 10 min and reacted with protein inhibitor for 20 min after washing. Finally, the cells were photographed, counted and recorded after staining with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis

Statistical Product and Service Solutions (SPSS) 24.0 software (IBM, Armonk, NY, USA) was utilized for statistical analysis. Differences between two groups were analyzed by using the Student's t-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). $P<0.05$ suggested that the difference was statistically significant.

Results

Differences in gene expressions between the control and model groups

The differences in gene expressions between control

Table 1. Primer sequences for PCR.

	Forward/reverse primer	Primer sequence
IP-10	Forward primer	CCACCTAGCTGTAGCAGACAC
	Reverse primer	AGGGCTCCTGCGTAGAAGTT
CXCR3	Forward primer	TTTGACCGCTACCTGAACATAGT
	Reverse primer	GGGAAGTTGTATTGGCAGTGG
IL-6	Forward primer	GGTCATGGCCTACTGCTATGC
	Reverse primer	CCACGTCTACCCTGCTTTCT
IL-12	Forward primer	GGTTAGTGAACGTCAAGTGCT
	Reverse primer	CCCCATAATCGTAGGGAGAGGT
TNF-α	Forward primer	TCTCCCTACGATTATGGGGAAAA
	Reverse primer	GGTTCTGTCAAAGTTCAGGCT
IL-1β	Forward primer	CTCTTTGCCCTCCCAGATTTC
	Reverse primer	GGCATAGCAGTAGGCCATGAC
GAPDH	Forward primer	TACCTTGAGGTTAGTGAACGTCA
	Reverse primer	CGCTCTCGTTTTCCCCATAATC

group and model group are shown in Figure 1 and Figure 2. It was indicated in volcano plot that there were some differentially expressed genes (fold change >1.2, P<0.01) in the retinal tissues of rats between control group and model group. Meanwhile, the heatmap displayed that there was a great difference in the gene expression patterns between control group and model group, and the gene expressions of IP-10 and CXCR3 in model group were remarkably higher than those in control group (P<0.05).

Enrichment analyses of differential genes in control and model groups

According to the GO enrichment analysis (Figure 3) and KEGG enrichment analysis (Figure 4) of differential genes in control group and model group, the differential genes in the two groups were apparently enriched in such processes as the cyclic adenosine monophosphate (cAMP) metabolic regulatory pathway, chemotaxis of immunocytes, proliferation of endothelial cells, response of cytokine receptors, apoptosis, mammalian target of rapamycin (mTOR) signaling pathway, and transforming growth factor-beta (TGF-β)-related signaling pathway.

Messenger RNA (mRNA) and protein expressions of IP-10 and CXCR3 in retinal tissues in control group, model group and inhibitor group

The mRNA and protein expressions of IP-10 and CXCR3 in retinal tissues in control group, model group and inhibitor group are displayed in Figure 5 and Figure 6, respectively. The differentially expressed IP-10 and CXCR3 in model group were screened out through sequencing, which were subsequently verified at the transcriptome and protein levels. Additionally, the expression of the IP-10/CXCR3 signaling pathway was repressed by inhibitor AMG 487. The mRNA expressions of IP-10 and

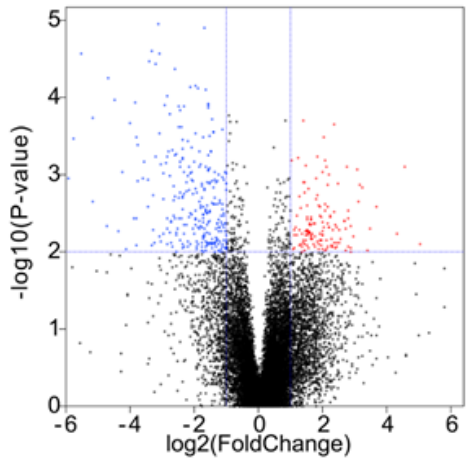

Figure 1. Volcano plot of differences in gene expressions between control group and model group.

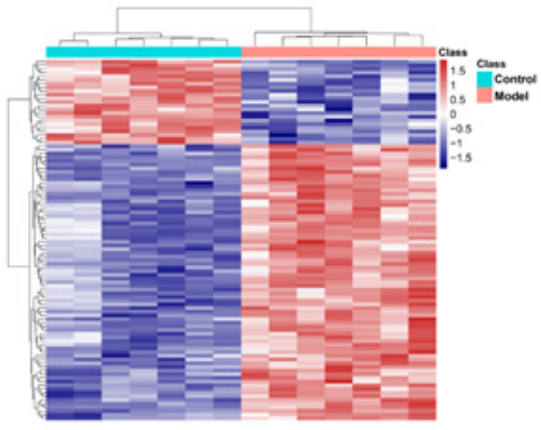

Figure 2. Heatmap of differences in gene expressions between control group and model group.

Figure 3. GO enrichment analysis of differential genes in control group and model group.

Figure 4. KEGG enrichment analysis of differential genes in control group and model group.

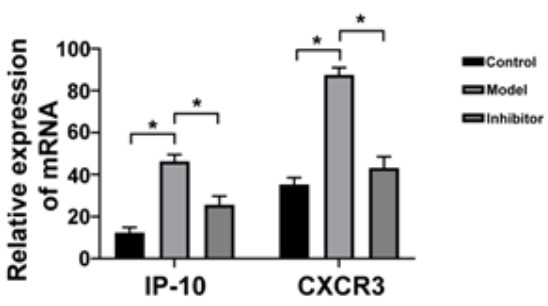

Figure 5. MRNA expressions of IP-10 and CXCR3 in retinal tissues in control group, model group and inhibitor group (*P<0.05).

CXCR3 in model group were obviously higher than those in control group (P<0.05), while they were notably lower in inhibitor group than those in model group (P<0.05). Besides, the protein levels of IP-10 and CXCR3 were identical to the mRNA levels.

Changes in apoptotic cells in rat retina in control group, model group and inhibitor group

Based on the changes in apoptotic cells in rat retina in control group, model group and inhibitor group (Figure 7), it was found that the number of apoptotic cells was increased markedly in model group compared with that in control group (P<0.05) and inhibitor group (P<0.05).

Expressions of inflammatory molecules in retinal tissues in control group, model group and inhibitor group

The results of the expressions of inflammatory molecules IL-6, IL-12, TNF-α and IL-1β in retinal tissues in control group, model group and inhibitor group (Table 2) manifested that model group exhibited evidently higher expression levels of IL-6, TNF-α and IL-1β in retinal tissues than control group (P<0.05), while inhibitor group had distinctly lower expression levels of IL-6, TNF-α and IL-1β in retinal tissues than model group (P<0.05).

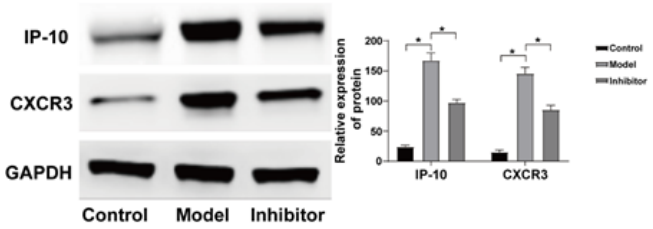

Figure 6. Protein expressions of IP-10 and CXCR3 in retinal tissues in control group, model group and inhibitor group (*P<0.05).

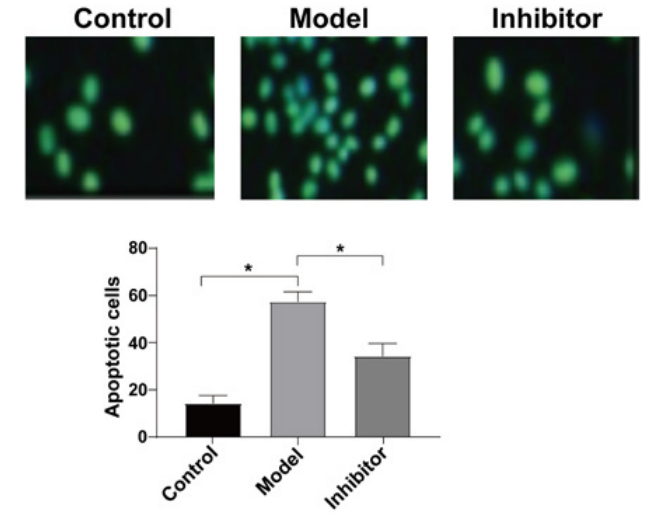

Figure 7. Changes in number of apoptotic cells in rat retina in control group, model group and inhibitor group (magnification: 400×) (*P<0.05).

Discussion

The incidence rate of diabetes mellitus is rising constantly with the continuous increase of population base and aggregation of population aging in the world, becoming one of the major diseases threatening human health (11). Patients with diabetes mellitus, a metabolic disease with multiple complications, are often accompanied by endocrine disorders, immune disorders and vasculopathy *in vivo* (12). Diabetes mellitus can trigger ocular complications such as cataracts and external ophthalmoplegia, but diabetic retinopathy is the most common (13). As an ocular complication of diabetes mellitus, diabetic retinopathy can impair patient's visual acuity and even lead to blindness, seriously reducing patient's quality of life (14). The occurrence of diabetic retinopathy may be associated with the disease progression and metabolic level in patients, which can be facilitated by relatively high blood pressure, lipid and glucose (15). Moreover, the pathogenesis of diabetic retinopathy is primarily correlated with local vascular endothelial injury in the eyes, where the injury probably results from the dysregulated expression of intra-cellular molecules and activated/inactivated relevant signaling pathways, thus increasing cell apoptosis and necrosis (16). Therefore, seeking new molecular pathogenesis of diabetic retinopathy can prominently help diagnose and treat the disease.

Signaling pathways are vital players in the pathogenesis of diseases and have correlations with various complex physiological and pathological pathways (17). Different from the expression of individual genes, the development of diseases can be researched at a relatively overall level of several molecules of signaling pathways. A variety of signaling pathways have been confirmed to be related to the development of diabetic retinopathy, including the TLR4/NF-κB signaling pathway (18) and the Hippo signaling pathway (19). IP-10, the key molecule of the IP-10/CXCR3 signaling pathway, is capable of stimulating the chemotaxis of lymphocytes and promoting the occurrence of Th1 inflammatory responses together with its receptor CXCR3. The activation of the signaling pathway can facilitate T cells to approach target tissues to perform their functions. Studies have testified that the IP-10/CXCR3 signaling pathway is associated with the incidence of diverse diseases such as eosinophilia (20) and hepatocellular carcinoma (21-26). In this research, the changes in gene expression patterns in rats with diabetic retinopathy were determined by means of sequencing, and the major related pathways to the complication were obtained *via* enrichment analysis after the differential genes were acquired. It was discovered that a part of genes were differentially expressed (fold change >1.2, P<0.01) in retinal tissues of rats between control group and model group, and the gene

Table 2. Expressions of inflammatory molecules in retinal tissues in control group, model group and inhibitor group.

Group	IL-6	IL-12	TNF-α	IL-1β
Control group	12.24±2.14	4.32±0.84	25.25±2.36	49.32±4.23
Model group	26.24±3.14[a]	4.72±0.64	56.19±4.21[a]	98.34±11.21[a]
Inhibitor group	15.34±2.14[b]	5.26±1.25	30.52±3.11[b]	42.12±4.26[b]

Note: [a]P<0.05 *vs.* control group, and [b]P<0.05 *vs.* inhibitor group, *t*-test.

expression patterns were distinctly different. These results suggest that great changes in gene expression are likely to occur in tissues with diabetic retinopathy, which may be the cause of retinal endothelial cell injury. Meanwhile, it was revealed in this research that model group had evidently higher gene expressions of IP-10 and CXCR3 than control group (P<0.05), implying that IP-10 and CXCR3 may play vital roles in the occurrence of diabetic retinopathy. Furthermore, enrichment analyses manifested that the differential genes in control group and model group were prominently enriched in the cAMP metabolic regulatory pathway, chemotaxis of immunocytes, proliferation of endothelial cells, response of cytokine receptors, apoptosis, the mTOR signaling pathway and TGF-β-related signaling pathway, illustrating that aggregation of immune cells and inflammation may be triggered in the tissues in diabetic retinopathy through the regulation of multiple intra-cellular signaling pathways such as the IP-10/CXCR3 signaling pathway, thereby exacerbating pathological changes in tissues.

As the sequencing results indicated that the IP-10/CXCR3 signaling pathway might have important effects in diabetic retinopathy, whether the expression of the molecules in each group of rats was consistent with the sequencing results was verified, and the expression of the IP-10/CXCR3 signaling pathway was suppressed using inhibitor AMG 487. It was found that the mRNA expressions of IP-10 and CXCR3 in model group were raised remarkably in comparison with those in control group (P<0.05), while they were reduced distinctly in inhibitor group compared with those in model group (P<0.05). Moreover, the same protein levels of IP-10 and CXCR3 were observed. All these results elucidate that the IP-10/CXCR3 signaling pathway is indeed activated in the tissues with diabetic retinopathy, which may be correlated with the disease progression.

According to in-depth experiments, model group had prominently more apoptotic cells than control group (P<0.05), and inhibitor group exhibited much fewer apoptotic cells than model group (P<0.05). The expression levels of IL-6, TNF-α and IL-1β in retinal tissues were elevated markedly in model group compared with those in control group (P<0.05), while they were lowered in inhibitor group in contrast with those in model group (P<0.05).

These findings imply that in the case of diabetic retinopathy, the IP-10/CXCR3 signaling pathway probably stimulates cell apoptosis and inflammatory responses to aggravate the disease. However, AMG 487, the inhibitor of the IP-10/CXCR3 signaling pathway, can inhibit the cell apoptosis and inflammatory responses enhanced by the pathway, so it may be a potential drug for diabetic retinopathy and needs to be studied by subsequent experiments.

Conflict of Interest

The authors declared no conflict of interest.

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